^{35}S-SULFIDE INCORPORATION DURING ALKALINE TREATMENT OF KERATIN AND ITS RELATION TO LANTHIONINE FORMATION

S. H. Feairheller, M. M. Taylor, and D. G. Bailey

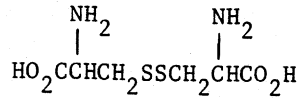
Eastern Regional Research Center, Agricultural Research Service, U.S. Department of Agriculture, Philadelphia, Pennsylvania 19118

ABSTRACT

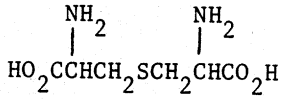
Cattle hair exposed to solutions of ^{35}S-sulfide ions at pH 12.5, hydrolyzed with acid, and analyzed for amino acids with simultaneous measurement of the radioactivity of the eluate from the analytical column showed 87 percent of the radioactivity incorporated in the hair in three amino acids: cysteic acid, lanthionine, and cystine. This and other evidence presented lend further support to the intermediacy of dehydroalanyl residues formed by a β-elimination reaction in the conversion of cystinyl residues to lanthionyl residues in proteins. The presence of radioactively labelled cystine in the hydrolyzate indicates that the dehydroalanyl residues are also capable of reforming cystinyl residues under these same conditions through a series of reversible reactions.

INTRODUCTION

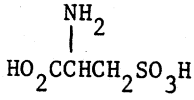
Conditions and reagents used to remove hair or wool from animal hides and skins in the manufacture of leather cause the same reactions of the sulfur-containing amino acids as those described in the papers both preceding and following this one in the Symposium. These alkaline solutions (pH 12.5) of sulfide ions convert cystinyl residues in the keratin to lanthionyl and/or lysinoalanyl residues, depending on the quantities of sulfide ions present. If sufficient sulfide ions are present most of the hair or wool is completely dissolved. If limited amounts are present



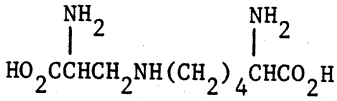
Cystine



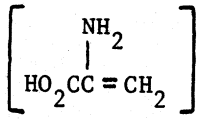
Lanthionine



Cysteic Acid



Lysinoalanine



Dehydroalanine

the hair or wool remains intact but its attachment to the hide or skin is considerably weakened, substantial conversion of the cystinyl residues to lanthionyl and lysinoalanyl residues takes place, and the hair or wool protein becomes more resistant to further attack by the reagents.

In early investigations, the formation of the lanthionyl residues was considered responsible for the increased resistance of the treated hair (Merrill, 1956; Windus and Showell, 1968). Subsequent observation that lysinoalanyl residues were also formed led to consideration of their involvement, the proposal that a β-elimination reaction is the initial step in these conversions,

and that dehydroalanyl residues are intermediates in the reactions (Feairheller et al., 1972). Further supporting evidence was obtained when the addition of mercaptans and secondary amines to these reaction mixtures resulted in the chromatographic detection of S-substituted cysteines and N-substituted β-aminoalanines (the products expected from the addition of these added compounds to the dehydroalanyl residues) in hydrolyzates of the hair (Feairheller et al., 1976). These authors also presented evidence suggesting the reversibility of these reactions or, at least, the interconversions of the sulfur-containing amino acids. This evidence and the results of our further investigations in this area are described in this report.

EXPERIMENTAL

Experiment Using $Na_2^{35}S$*

Five mg of thoroughly washed and solvent degreased cattle hair, 8 mg of $Ca(OH)_2$, and 0.9 ml of water were placed in a glass hydrolysis tube and to this was added 0.1 ml of a solution of 0.5 mg of $Na_2\ ^{35}S$ (specific activity 9.2 mCi/mM)**. The resulting mixture was allowed to stand for three days. The solution was then removed from the residual hair and the hair was washed ten times with distilled water using a pipette and without removing the hair from the tube. The hair was washed once with ten percent hydrochloric acid solution and finally 2.5 ml of 6N hydrochloric acid solution were added. The contents of the tube were frozen in a dry ice-acetone bath, the tube was sealed under vacuum and the sealed tube and contents were then heated at 105°C for 18 hr. The tube was opened and the contents were evaporated to dryness under a stream of nitrogen. The residue was taken up in exactly 1 ml of 0.1N hydrochloric acid solution for analysis. The solution was analyzed for amino acid composition on a single column system (Piez and Morris, 1960) using a continuous gradient elution buffer. A stream splitting device was used to divert 25% of the eluate from the analytical column through the normal analyzer detection system. The remaining 75% simultaneously flowed through the detector of a liquid scintillation system. The overall system

---

*Experimental conditions for all other reactions discussed can be found in Feairheller et al., 1972; and Feairheller et al., 1976.

**Caution must be exercised in the use of sulfides because of the toxic nature of the gas (H_2S) generated on acidification. All radioisotopes must be handled in accordance with prescribed AEC guidelines for safe use.

was precalibrated with known samples of radioactively labelled amino acids. Excellent correlation was obtained between the elution times of the amino acids and the peaks of radioactivity. Fractions of the effluent from the scintillation counter containing the radioactivity were mixed with solutions of the appropriate amino acid and rechromatographed to further substantiate the correlation.

RESULTS AND DISCUSSION

Previous work (Feairheller et al., 1972; Feairheller et al., 1976) indicated that the addition of sulfide ions to these reaction mixtures of alkali-treated hair keratin had a threefold effect which was contradictory to the sole involvement of lanthionine in this increase in the stability of the hair protein. Our present study confirmed this effect (Table 1). First, the addition of sulfide ions caused a decrease in the resistance of the hair to further dissolution. This effect is well known to leather chemists (sodium sulfide is usually added to "unhairing" baths prior to addition of the alkali). Second, the amount of lanthionine formed increased in the presence of added sulfide ions. In some cases the amount actually doubled. Finally, the amount of lysinoalanine formed decreased to a trace or less in the presence of added sulfide ions. The amount of cystine lost from the hair keratin as a result of these treatments did not vary greatly, regardless of whether sulfide ions were present or not. Clearly, all of these treatments render the hair considerably more resistant to further dissolution; however, there is a significant two- or threefold difference between those samples exposed to sulfide ions and those not exposed.

We determined what the involvement of the sulfide ion was in these reactions by using ^{35}S-sulfide in the reaction mixtures, isolating the exposed hair, hydrolyzing it in preparation for amino acid analysis, chromatographically analyzing the hydrolyzate, and measuring the amino acid content and radioactivity simultaneously using a stream-splitting device on the analyzer (Table 2).

The lanthionine contained a substantial amount of ^{35}S, indicating that the reactions by which it was formed permitted the incorporation of sulfide ion or some other form of sulfur derivable from sulfide ion under these conditions. Its specific activity was about 45% that of the added sulfide. The oxidized product, cysteic acid, had by far the highest specific activity, approaching (94%) that of the added sulfide and indicating that the reactions leading to its formation involve exclusive incorporation of sulfur in some form from solution, rather than from sulfur originally present in the hair keratin. This is especially significant since cysteic acid is a final product in the reaction sequence.

TABLE 1

Crosslinking Amino Acid Content of Alkali-Treated Hair Keratin and Resistance to Further Degradation

Alkali Treatment			Amino Acids (As % of Original Cystine)				Solubility[e]
Reagent(s)[a]	Time (Days)	pH	$(Cys)_2$	Lan[b]	Lya[c]	Total	(As % of Sample Exposed)
Control	-	-	100	0	0	100	81
$Ca(OH)_2$	1	12.4	37	30	4	71	6
$Ca(OH)_2$	5	12.4	26	38	8	72	5
$Ca(OH)_2 + Na_2S$	3	12.4	25	59	0	84	10
$Ca(OH)_2 + Na_2S$[d]	3	12.4	23	55	0	78	18
NaOH	1	12.6	51	25	4	76	6
$NaOH + Na_2S$	1	12.0	47	38	0	85	11

[a]Refer to Feairheller et al. (1972) for experimental details. [b]Lanthionine. [c]Lysinoalanine. [d]Double amount of Na_2S. [e]Hair recovered from alkali treatments weighed and shaken vigorously in a solution which was 2% by weight of sodium hydroxide and 1.5% by weight of sodium sulfide at 40°C for one hr. Remaining hair was recovered, washed, dried and weighed.

Table 2

Distribution of Sulfur-Containing Amino Acids and Radioactivity

Amino Acid	Quantity	Specific[a] Activity
	(mM)	(mCi/mM)
Cystine	.32	3.66
Lanthionine	.56	4.18
Cysteic Acid	.18	8.67

[a]Specific activity of added $Na_2^{35}S$:9.2mCi/mM.

While not as heavily labelled as the other two amino acids, the cystine itself contained a significant amount of ^{35}S, about 40% of the specific activity of the added sulfide. The sequence of reactions involved in its decomposition permits its reformation from some intermediate which has acquired sulfur from solution. Its reformation could take place by a series of reversible reactions or a separate sequence.

A group of reactions, some of which can be used to explain these results, is set forth in equations 1-8. The evidence is overwhelming (Danehy, 1971) that the β-elimination reaction resulting in the formation of dehydroalanyl residue, equation 1 is the initial reaction. This residue can take part in a number of

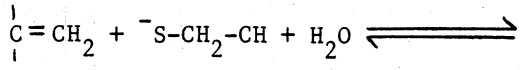
$$HO^- + H\text{-}\overset{|}{C}\text{-}CH_2\text{-}S\text{-}S\text{-}CH_2\text{-}\overset{|}{C}H \rightleftharpoons H_2O + \overset{|}{C}=CH_2 + {}^-S\text{-}S\text{-}CH_2\text{-}\overset{|}{C}H \quad (1)$$

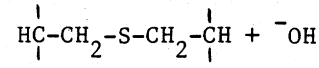
$$HO^- + H\overset{|}{C}\text{-}CH_2\text{-}S\text{-}S^- \rightleftharpoons H_2O + \overset{|}{C}=CH_2 + S_2^= \quad (2)$$

$$H\overset{|}{C}\text{-}CH_2\text{-}S\text{-}S^- \rightleftharpoons H\overset{|}{C}\text{-}CH_2\text{-}S^- + S^\circ \quad (3)$$

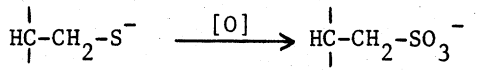
$$S^= + S^\circ \rightleftharpoons S_2^= \quad (4)$$

$$\overset{|}{C}=CH_2 + S^= + H_2O \rightleftharpoons H\overset{|}{C}\text{-}CH_2\text{-}S^- + {}^-OH \quad (5)$$

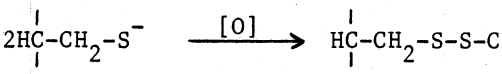




(6)



(7)



(8)

addition reactions, including the reformation of cystine by the reverse of this reaction by which it is formed. In the intact hair keratin, the reversibility of the reaction would be enhanced by the close proximity of the two residues. Other reactions of the dehydroalanyl residues are illustrated in equations 5 and 6. An additional reaction, not shown, is the formation of lysinoalanyl residues by reaction with nearby lysinyl residues.

Equations 2 and 3 illustrate alternative modes of decomposition of the second product of the reaction in equation 1. Neither equation 2 nor 3 is known to have any precedent. However, if they do occur and are reversible, they would account for the incorporation of either ionic or elemental sulfur from solution. These forms of sulfur are in equilibrium by way of the reaction shown in equation 4.

The addition of sulfide ion to a dehydroalanyl residue, equation 5, is to be expected under these conditions, as is the addition of the cysteinyl residue anion, equation 6. This sequence would obviously lead to the incorporation of the added sulfide ion into lanthionine, and its presence might be expected to compete very well with lysinyl residues in the addition reactions and thus hinder or limit the formation of lysinoalanyl residues. Only a finite number of the lysinyl residues, which are fixed in the protein, are available, while the sulfide ions are present in excess and free in solution. Nothing is known about the reversibility of equation 5 at this time. Evidence for the reversibility of the reaction in equation 6 is given later. The β-elimination reaction of a lanthionyl residue, the reverse of equation 6, would indicate that it is not the stable crosslink it is commonly thought to be (Danehy, 1971). The reversal of the reaction in equation 5 is unlikely, since it results in the accumulation of a second negative charge on an atom already containing one full negative charge. At pH 12.5, the sulfur would not be protonated and this elimination reaction is unlikely. A similar argument could be used against the forward reaction in equation 2, but in this case the negative charge is spread over two sulfur atoms.

Excess sulfide ions would strongly favor the forward reaction of equation 5 as opposed to the reverse reaction, regardless of the other factors involved, and could successfully compete with the cysteinyl residue anions in the addition reactions thus preventing the formation of lanthionyl residues, equation 6. However, as dictated by their mode of formation, the dehydroalanyl residues and cysteinyl residue anions are in close proximity to each other in the intact protein and are thus able to overcome the excess sulfide ions. Therefore the reaction in equation 6 takes place. This was not the case with the lysinoalanine formation where the sulfide ions successfully competed with the lysinyl residues and prevented the formation of lysinoalanyl residues.

These six reactions, in appropriate combination, along with the oxidation of the cysteinyl residues to cysteic acid residues, equation 7, would account for the ^{35}S incorporation found.

An alternative route to the formation of cystine that would also account for the incorporation of ^{35}S from sulfide ions in solution is the oxidative reaction, equation 8. Oxidation is taking place as indicated by the formation of cysteic acid. The reactions in equation 1, 5, 6, 7, and 8 could then account for the different products, but not the ^{35}S incorporation into lanthionine. This sequence of reactions would have resulted in the formation of lanthionine with a specific activity approaching that of the added sulfide, and this was not found. The reaction in equation 3 is almost certainly involved, and the lanthionine is formed from cysteinyl residue anions produced by this reaction as well as by the addition of sulfide to a dehydroalanyl residue, equation 5.

Therefore, the reactions most likely involved are those shown in equations 1, 3, 4, 5, 6, and 7, with the reactions shown in equations 1, 3, and 4 being completely reversible under the conditions that we used. The reactions shown in equations 2 and 8 are not as likely and are not necessary to explain the results. It is also unlikely that the reaction in equation 5 is reversible.

Finally, evidence presented by Feairheller et al. (1976) bears on the reversibility of the reaction in equation 6. Feairheller et al. (1972) had shown that the conversion of cystinyl residues to lanthionyl residues in proteins was effected by sodium hydroxide solutions with a pH of about 12.5 as well as by saturated solutions of calcium hydroxide, commonly used in "unhairing" reactions at the same pH. However, Danehy (1971) concluded that cystine, the free amino acid, can not be converted to lanthionine under these same conditions. This appears to be substantiated on theoretical grounds. In the free amino acid, the amino group and negatively charged carboxylate anion attached to the α-carbon atom generate a high electron density and prevent the abstraction of the α-hydrogen atom by base. Exposure of cystine to solutions of

lithium, sodium, or potassium hydroxide at a pH of about 12.5 caused no reaction over a period of several days. However, exposure of cystine to a solution of either calcium or strontium hyroxide at the same pH resulted in reaction of the cystine and formation of lanthionine. Feairheller et al. (1976) suggested that these divalent metal ions are capable of forming complexes of sufficient stability with the cystine, involving the carboxylate anions and α-amino groups, to reduce the electron density about the α-carbon atom and thus permit the β-elimination reaction to take place. Magnesium and barium hydroxides were not effective. The lanthionine accounted for one-third of the cystine in these reaction mixtures after 48 hr but then it started to disappear. The cystine was completely consumed within 14 days and the lanthionine within 28 days. Other products detected were alanine, cysteic acid, ammonia, hydrogen sulfide, and 2-methylthiazolidine-2,4-dicarboxylic acid (Dann et al., 1957).

We next exposed lanthionine to the same conditions with calcium hydroxide and, as was indicated by the results of the previous experiments, it decomposed but did so initially at a much slower rate. After two days exposure, 86% of the lanthionine was recoverable. However, cystine was also present to the extent of 5% of the amount of the starting lanthionine or 36% of the lanthionine which had reacted. After seven days' exposure the amount of lanthionine recoverable had dropped to 20% of its original value and no cystine was detectable. The different metallic hydroxides had the same effect with lanthionine as they had with cystine. Calcium and strontium hydroxides caused the decomposition of lanthionine, while lithium, sodium, potassium, magnesium, and barium hydroxides did not.

There is no reason to suppose that a mechanism other than the β-elimination reaction is operative here and, if this is the case, it must be concluded that lanthionyl residues in proteins are capable of the same reactions. The reaction shown in equation 6 is therefore reversible, and one or both products of the reverse reaction are capable of being converted to cystine.

CONCLUSIONS

The involvement of the β-elimination reaction and the intermediacy of dehydroalanyl residues in reactions of hair keratin are further substantiated by the results of this study. The vulnerability of lanthionine to the same conditions adds still further support and indicates that this residue in proteins is not a completely inert crosslink. Its reaction in proteins through the elimination type mechanism is implied. The lanthionine was found to be more inert to these conditions than the cystine and did increase the

stability of the treated hair but, not unexpectedly, the lysinoalanine appears to represent the most inert of those crosslinks known to be present. It is also reasonable, in view of the reaction sequence discussed, that added sulfide ions should compete with lysinyl residues and favor formation of lanthionyl residues at the expense of lysinoalanyl residues.

REFERENCES

Danehy, J. P. (1971). The alkaline decomposition of organic disulfides and related nucleophilic displacements of sulfur from sulfur. Int. J. Sulfur Chem. B., 6, 103.

Dann, J. R., Oliver, G. L., and Gates, J. W. (1957). The reaction of cystine and lanthionine with aqueous calcium hydroxide. The identification of 2-methylthiazolidene-2,4-dicarboxylic acid. J. Am. Chem. Soc., 79, 1644.

Feairheller, S. H., Taylor, M. M., Filachione, E. M., and Windus, W. (1972). New amino acids formed in hair during unhairing. J. Am. Leather Chem. Assoc., 67, 98.

Feairheller, S. H., Taylor, M. M., Bailey, D. G., and Windus, W. (1976). Further evidence in support of the elimination reactions as the mechanism of alkaline unhairing. J. Am. Leather Chem. Assoc., in press.

Merrill, H. B. (1956). "Chemistry and Technology of Leather." Vol. II, Chapter 8 (O'Flaherty, F., Roddy, W. T., and Lollard, R. M., eds.), Reinhold Publishing Corp., New York, NY.

Piez, K. A., and Morris, L. (1960). A modified procedure for the automatic analysis of amino acids. Anal. Biochem., 1, 187.

Windus, W., and Showell, J. S. (1968). An interpretation of the mechanism of unhairing as a nucleophilic displacement. J. Am. Leather Chem. Assoc., 63, 258.